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A tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P450s including the polymorphic human cytochrome P4502D6

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We have developed a human B-lymphoblastoid cell line, designated 2D6/Hol, which stably expresses human cytochrome P450 CYP2D6 cDNA. This cell line exhibits bufuralol 1'-hydroxylase activity and immunologically detectable CYP2D6 protein. The specific activity of (+)-bufuralol 1'-hydroxylase in microsomes from 2D6/Hol cells was comparable to that observed in human liver microsomes. This cell line was used to examine the mutagenicity activation of three tobacco smoke-derived nitrosamines, *N*-nitrosonornicotine (NNN), 1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal) (NNA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), by CYP2D6. Exposure of 2D6/Hol cells to NNK concentrations of 30–90 μg/ml induced a concentration-dependent decrease in relative survival and increase in mutant fraction at the hypoxanthine guanine phosphoribosyl transferase (hprt) locus. In contrast, NNK was non-mutagenic and non-cytotoxic to control cells at exposure concentrations up to 150 μg/ml. NNK mutagenicity in 2D6/Hol cells was compared to the responses observed in isogenic cell lines expressing human CYP1A2 (1A2/Hol), human CYP2A3 (2A3/Hol) and human CYP2E1 (2E1/Hol). These three additional human cytochrome P450-expressing cell lines were also found to be sensitive to NNK-induced mutagenicity and cytotoxicity. We found no evidence for CYP2D6-mediated activation of NNN or NNA. NNN was non-cytotoxic and non-mutagenic to both control and 2D6/Hol cells. NNA was equally cytotoxic and mutagenic to control cells and 2D6/Hol cells. The activation of NNA to a mutagen may have been carried out by P450 native to the AHH-1 TK+/– cell line. The 2D6/Hol cell line, in conjunction with the control cell line and other isogenic cell lines expressing other human cytochrome P450 cDNAs provides a useful system for the examination of the role of the polymorphic CYP2D6 in human procarcinogen activation and drug metabolism.

Introduction

Cytochromes P450 are a superfamily of hemoproteins, many of which can metabolize xenobiotics such as procarcinogens, drugs and environmental pollutants. The expression of some human cytochrome P450s has been found to be polymorphic. Population studies with the anti-hypertensive drug, debrisoquine, have revealed that 5–10% of the Caucasian population in Europe and North America poorly metabolize this drug (1,2). The poor metabolizer phenotype behaves as an autosomal recessive trait and is also associated with deficient metabolism of more than 20 drugs in common use (3).

A cDNA containing the full cytochrome P450, CYP2D6, coding sequence has been isolated and the molecular nature of the poor metabolizer genotype has been characterized (4). Poor metabolizers contained mutations in the CYP2D6 gene which primarily result in aberrant mRNA splicing. The aberrant mRNA cannot produce functional protein.

Recent epidemiological studies have suggested an increased lung cancer risk in smokers with the ability to metabolize extensively debrisoquine (5–7). Poor metabolizers of debrisoquine were found to be at a 6-fold lower risk for the development of lung cancer compared to extensive metabolizers. The mechanism for this lower risk among poor metabolizers remains obscure. One potential mechanism is through a reduced ability to perform CYP2D6-catalyzed activation of tobacco smoke-derived procarcinogens. However, unlike most of the human cytochrome P450s, CYP2D6 has not been reported to activate any procarcinogens.

We have developed several human cell lines that stably express human cytochrome P450 cDNAs (CYP1A2, CYP2A3, CYP3A4 and CYP2E1) (8–10). The AHH-1 TK+/– human B-lymphoblastoid cell line, which was used in these studies, is a convenient vehicle to measure the induction of gene mutations at the hypoxanthine phosphoribosyl transferase (hprt*) and thymidine kinase loci and also the induction of chromosomal aberrations and aneuploidy. This cell line has some native CYP1A1 activity, which is inducible by polycyclic aromatic hydrocarbon-type inducers. These cDNA-expressing cell lines have been utilized to analyze procarcinogen activation, and metabolism by specific human cytochrome P450s.

Here we report the development of a human cell line stably expressing CYP2D6. The cell line, designated 2D6/Hol, metabolizes bufuralol, a prototypic substrate for CYP2D6 and contains immunologically detectable CYP2D6 protein. We have used this cell line to screen three tobacco smoke-specific nitrosamines, 1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal (NNA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN), for activation to mutagenic metabolites by CYP2D6.

The prevalence and importance of tobacco smoke-specific nitrosamines have been reviewed (11,12). Among this class of chemicals, the most potent carcinogen appears to be NNK, which has been found to induce tumors in rats, mice and hamsters. The primary route of metabolic activation appears to be α-carbon hydroxylations which lead to an unstable diazohydroxide intermediate. NNK has the potential to be metabolized to a methylating agent and pyridyloxobutylating agent. NNK-derived adducts from DNA methylation (13) and DNA pyridyloxobutylation (14) have been reported. NNN is a less potent rodent carcinogen than NNK (15) while NNA was not found to be carcinogenic to A/J mice (16).

*Abbreviations: hprt, hypoxanthine guanine phosphoribosyl transferase; NNA, 1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosonornicotine; HSVtk, Herpes Simplex virus thymidine kinase; NDMA, *N*-nitrosodimethylamine.

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Materials and methods

Cells, tissue culture, DNA introduction

AHH-1 TK+/− is a human B-lymphoblastoid cell line. Cells were maintained in RPMI medium 1640 supplemented to 9% (v/v) with horse serum. Protoplast fusions were performed according to Yoakum (17). Cells bearing recombinant plasmids were maintained in medium without histidine and containing 2 mM l-histidinol (pEBVHistk-based vectors).

Enzymes and reagents

Restriction endonucleases, Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs. RPMI medium 1640, benzo[*a*]pyrene and l-histidinol were purchased from Sigma Chemical Company, St Louis, MO. NNA, NNK and NNN were obtained from the NCI Chemical Carcinogen Repository. (+)-Bufuralol, debrisoquine, 4-hydroxy-debrisoquine and carbinol were gifts from Dr Urs Meyer, Basel, Switzerland.

Cytotoxicity and mutagenicity assays

Cytotoxicity was estimated by measuring growth after treatment. After cultures have resumed exponential growth, the cumulative growth of the mutagen-treated cultures was divided by the cumulative growth of the negative control cultures to yield relative survival. Induction of mutation at the hprt locus was measured by previously published protocols (18) with minor modifications. Human lymphoblasts were exposed to the mutagen for 28 h. Each replicate culture contained 3×10^7 cells. After a 7 day phenotypic expression period, the mutant fraction was measured by plating 7.5×10^6 cells in three 96 well microtiter plates in the presence of 6-thioguanine (0.6 μg/ml) and 500 cells in two 96 well microtiter plates without selection. NNK, NNN and NNA were dissolved in water for delivery to the cell cultures.

DNA manipulations

Bacterial transformations, plasmid DNA isolations and construction of the expression vectors were performed according to standard protocols (19).

Preparation of microsomal samples

Cell suspensions were harvested by centrifugation at 1000 *g* for 5 min and resuspended in deionized water at a concentration of 0.33 g cells/ml. The suspension was homogenized by 15 strokes in a glass homogenizer. Potassium phosphate (0.05 M, pH 7.4) was then added to yield a final concentration of 0.1 M. The cell homogenate was sonicated briefly and rehomogenized. The homogenate was centrifuged at 9000 *g* for 10 minutes at 4°C. The pellets were resuspended in 0.1 M potassium phosphate (pH 7.4) and centrifuged again at 9000 *g* for 10 min. The supernatants from both centrifugation steps were combined and centrifuged at 100 000 *g* for 10 min at 4°C. Longer 100 000 *g* centrifugations were found to result in substantially lower specific activities in the microsomes. The pellet containing the microsomal fraction was resuspended by homogenization with 0.1 M potassium phosphate (pH 7.4). Microsomal protein concentrations were determined by the method of Lowry *et al.* (20) using bovine serum albumin as the standard.

Enzyme assays

Bufuralol 1′-hydroxylase activity was measured according to (21) with the sole modification that the perchloric acid concentration in the HPLC solvent was 1 mM. Cells were incubated in 0.1 M potassium phosphate (pH 7.4) with 0.1 mM bufuralol at a final concentration of 4×10^6 cells/ml for 15 min. Microsomes were assayed at 0.5−1 mg protein/ml.

Western immunoblot analysis

Samples of total cell lysate were subjected to SDS−PAGE according to the method of Laemmli (22) and transferred to nitrocellulose membranes using an electrotransfer blotter. The filters were incubated with human antibody LKM-1, specific to CYP2D6 (23). Blots were developed with phosphatase-conjugated goat anti-human IgG.

Results and discussion

Development of the cell line

A cell line expressing CYP2D6 was constructed by transfecting a complete human CYP2D6 cDNA (4) in a pEBVHistk-derived expression vector under the control of the Herpes Simplex virus thymidine kinase (HSVtk) promoter and polyadenylation signal. This vector confers resistance to l-histidinol and contains the OriP sequences derived from Epstein−Barr virus which allows stable replication as extrachromosomal DNA in EBV-transformed lymphoblasts (24), such as AHH-1 TK+/−. The vector was introduced via protoplast fusion and a bulk population resistant to 0.5 mM l-histidinol was isolated. The l-histidinol concentration was gradually increased to 2 mM and a clonal population resistant to 2 mM l-histidinol was isolated.

Transfected cells expressing the CYP2D6 cDNA exhibited immunologically detectable CYP2D6 which catalyzed bufuralol 1′-hydroxylation (Figures 1 and 2). The enzymatic activity was stable for at least 90 days in cell culture. Kinetic analysis of (+)- and (−)-bufuralol hydroxylation indicated that the (+)-isomer had a V_{max} approximately twice that of the (−)-isomer (6.3 versus 3.2 pmol product/10^6 cells/min). The apparent K_ms for both isomers were $8-9 \times 10^{-6}$ M (Figure 3). The K_m value and relative V_{max} for the (+) and (−)-stereoisomers are consistent with those reported for these substrates in human liver microsomes (21). Our observed K_m was slightly higher than that

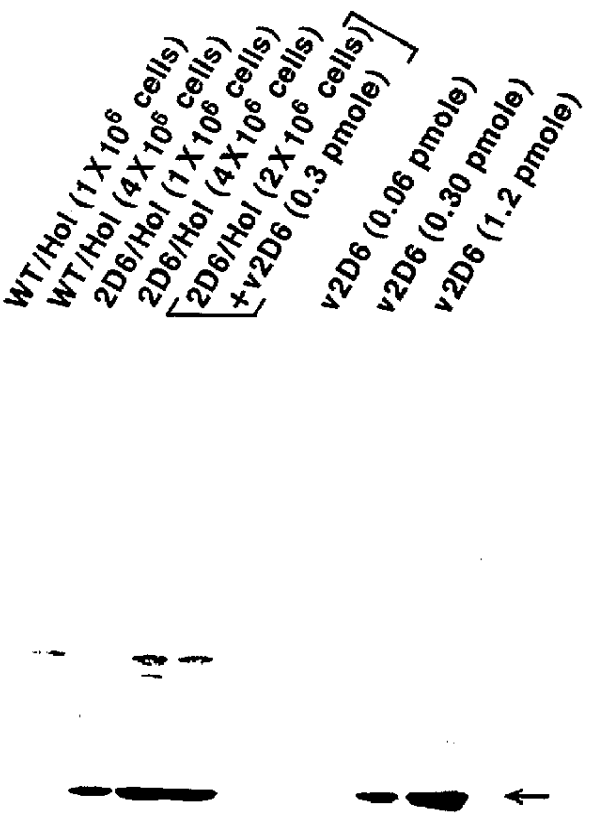

Fig. 1. Western immunoblot of total cell lysates. The 2D6/Hol cells analyzed contained (+)-bufuralol 1′-hydroxylase activity of 5 pmol/10^6 cells/min. WT/Hol cells did not contain detectable (+)-bufuralol 1′-hydroxylase activity (<0.05 pmol/10^6 cells/min). 2D6/Hol cells contained ~0.3 pmol CYP2D6 protein per 10^6 cells. Based on the specific activity, the turnover number for (+)-bufuralol was 15 min^{-1}. WT/Hol cells did not contain detectable CYP2D6 protein. Quantitation was achieved by comparison to spectrophotometrically quantitated CYP2D6 protein produced with a vaccinia virus vector.

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observed in human liver and the difference may be due to transport of bufuralol across the cell membrane.

The levels of CYP2D6 and (+)-bufuralol 1′-hydroxylase activity were increased ~3-fold after pretreatment with the combination of 0.2% DMSO and 4% horse serum. This increase in activity was time dependent and is inhibited by cycloheximide but not by actinomycin D (Table I), suggesting that translation and not transcription is involved. Similar pretreatment of cells expressing other cytochrome P450 cDNAs (CYP2A3 or CYP1A2) driven by the same promoter (HSVtk) did not result in an increase in enzymatic activity in whole cells. These results indicate that the increased CYP2D6 activity via treatment with DMSO and horse serum is probably due to sequences within the cDNA and not the HSV-tk promoter.

(+)-Bufuralol 1′-hyroxylase activity was also measured in microsomal fractions derived from 2D6/Hol cells pretreated with DMSO (0.2%) and horse serum (4%). The heme precursor, 4-aminolevulinic acid (30 μg/ml), was also added during induction to help support heme synthesis. The addition of 4-aminolevulinic increased (+)-bufuralol 1′-hydroxylase activity 20–25%. Microsomes were prepared with a yield of 7–8 mg protein/g wet wt cell pellet (2.5–3 g cells were obtained/l of culture). The (+)-bufuralol 1′-hydroxylase activity in the microsomes derived from 2D6/Hol cells was 470 ± 30 pmol/mg protein/min (mean ± SD, $n = 3$). (+)-Bufuralol 1′-hydroxylase activity was not

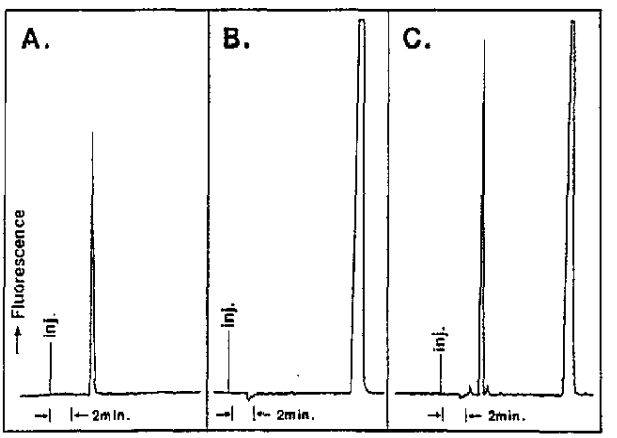

Fig. 2. High-performance liquid chromatograms of incubations of (+)-bufuralol with microsome preparations. Incubations and metabolite quantitation were carried out as described in Materials and methods. (A) Carbinol metabolite standard (114 pmol). (B) Twenty microliters of an incubation of control microsomes (1 mg/ml protein, incubated for 30 min). (C) Ten microliters of an incubation of CYP2D6-containing microsomes (1 mg/ml protein, incubated for 30 min).

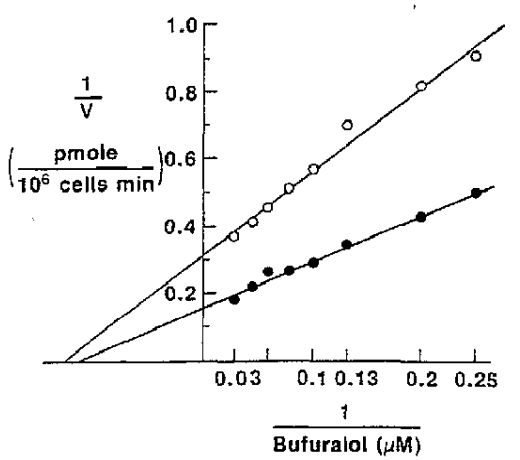

Fig. 3. Kinetic analysis of (+)- and (−)-bufuralol hydroxylation in whole 2D6/Hol cells. Incubations and metabolite quantitation were carried out as described in Materials and methods. Values are the mean of duplicate determinations. Kinetic values were obtained by linear regression analysis. (+)-Bufuralol: K_m = 8.7 μM, V_{max} = 6.3 pmol/10^6 cells/min; (−)-bufuralol K_m = 8.0 μM, V_{max} = 3.2 pmol/10^6 cells/min.

Table I. (+)-Bufuralol 1′-hydroxylase activities in whole cells

Condition	(+)-Bufuralol 1′-hydroxylase activity (pmol/10^6 cells/min)
Control	3.3 ± 1.3 (n = 9)
0.2% DMSO + 4% horse serum	9.8 ± 1.0 (n = 13)
0.2% DMSO + 4% horse serum 5 μg/ml actinomycin D	7.7 ± 0.3 (n = 2)
0.2% DMSO + 4% horse serum 5 μg/ml cycloheximide	3.8 ± 0.2 (n = 2)

Cells were incubated under the conditions described in the table for 16 h and (+)-bufuralol 1′-hydroxylase activity was measured in whole cells (19). Values are means ± SD.

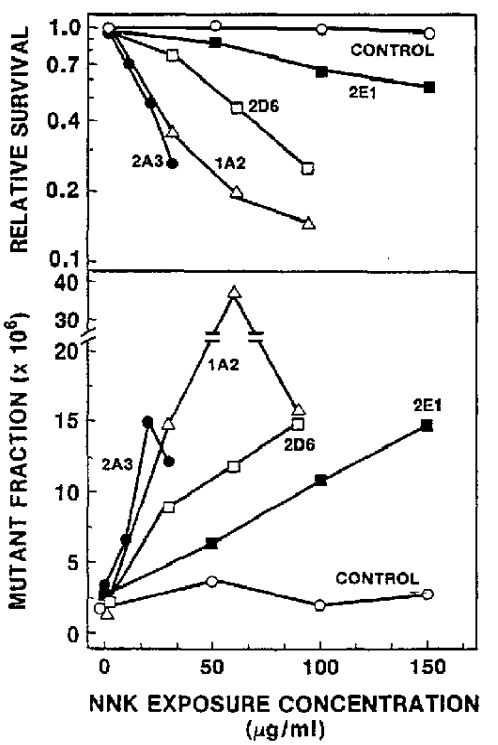

Fig. 4. Cytotoxicity and mutagenicity of NNK. Human lymhoblasts were exposed to NNK for 28 h and mutant fractions were measured at the hprt locus according to the methods previously described (18). Cytotoxicity was estimated by analyzing growth after treatment. Each cell line was tested in at least two independent experiments. Duplicate cultures were used at each exposure concentration. Mean values are plotted. The levels of CYP2D6 in 2D6/Hol cells were increased by pretreatment for 16 h with 0.2% DMSO and 4% horse serum. Symbols are as follows: open circles, control cells; closed circles, 2A3/Hol cells; open squares, 2D6/Hol cells; closed squares, 2E1/Hol cells; and open triangles, 1A2/Hol cells.

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detectable in control cell microsomes (<0.2 pmol/mg protein/min). This specific activity is within the range observed in human liver microsomes for extensive metabolizers of debrisoquine (4). Microsomes derived from 2D6/Hol cells were also found to hydroxylate debrisoquine. Control cell microsomes did not detectably metabolize debrisoquine.

Mutagenicity of NNK

We examined the cytotoxicity and mutagenicity of the tobacco smoke-derived mutagen NNK in cells expressing CYP2D6 and control (untransfected) cells (Figure 4). NNK was non-cytotoxic and non-mutagenic to control cells. In contrast, exposure of 2D6/Hol cells to 30−90 μg/ml NNK induced a concentration-dependent decrease in relative survival and increase in mutant fraction at the hprt locus. The mutagenic response observed for NNK in 2D6/Hol cells indicates that CYP2D6 activates this nitrosamine. CYP2A3 and CYP2E1 have been previously demonstrated to activate nitrosamines in this system (8,10). We therefore examined NNK mutagenicity in isogenic cell lines expressing CYP2A3 (2A3/Hol), CYP2E1 (2E1/Hol). We have also developed a cell line expressing CYP1A2 (1A2/Hol) which was also examined. All three additional cDNA-expressing cell lines were found to be sensitive to NNK-induced cytotoxicity and mutagenicity. The relative sensitivities of the cell lines to NNK was 1A2/Hol = 2A3/Hol > 2D6/Hol > 2E1/Hol (Figure 4).

Based on quantitative Western blots, calculations from specific enzymatic activities, and the turnover numbers for the specific substrates, 1A2/Hol cells, 2A3/Hol cells and DMSO/horse serum pretreated 2D6/Hol cells have comparable P450 contents with 0.7−1.2 pmol P450/10^6 cells. This represents an 8-fold increase over those reported for 2A3/Hyg and 1A2/Hyg (25) and reflects the use of a vector with 8-fold higher copy number. 2E1/Hol cells contain 5−10 times less P450. Therefore, the somewhat lower cytotoxicity and mutagenicity of NNK to 2E1/Hol cells is probably due to the lower P450 content. Normalization of the data from 2E1/Hol cells for P450 content would yield a mutagenic response more similar to the other transfected cell lines.

Activation of NNK to cytotoxic and mutgenic species appears to be associated with more than one cytochrome P450 form; each of four P450 forms were capable of activating this carcinogen. The relative contribution of these forms to NNK activation in human lung, the target tissue involved in increased risk, may depend on the relative abundance of the catalytically active P450 enzymes in the specific target cells in the lung.

Analysis of human cell growth during promutagen treatment can reveal some information regarding the nature of the DNA alkylating species. Human cells have a unique cell progression after exposure to lethal concentrations of low mol. wt alkylating agents (such as methylating agents). Cell populations receiving treatments that reproductively kill the vast majority of cells will progress to divide once prior to ceasing division (26). Exposure to lethal concentrations of agents that produce bulky adducts immediately stops cell progression. NNK is an asymmetrical nitrosamine with the potential to produce methyl or pyridyloxobutyl (13,14) adducts depending on how NNK is oxidized. Therefore, analysis of cell growth during the 28 h exposure can suggest which potential adduct is most prevalent. All cells exposed to all NNK exposure concentrations grew as well as negative control cells during treatment. Some of these NNK treatment concentrations were lethal to 80% of the cells. This observation suggests that the predominant cytotoxic alkylating species is small and probably the methyl group. Of course, direct analysis of DNA adduction is necessary to determine which DNA adducts are present in NNK-exposed cells.

Compared to *N*-nitrosodimethylamine (NDMA) in 2E1/Hol (10), NNK was ~1000-fold less active as a mutagen on a molar basis in 2D6/Hol, 2A3/Hol, 1A2/Hol and 2E1/Hol cells. If the alkylating species is the same for NNK and NDMA (a methylating moiety), the relative sensitivity to NNK and NDMA indicates that a very small percentage (on the order of 0.1%) of NNK was metabolized through the activating pathway. The unambiguous detection of NNK activation in this system is testimony to the sensitivity and versatility of human cell mutation assays as indicators of metabolic activation. We have previously reported 2A3/Hol cells and 2E1/Hol cells to be sensitive to the cytotoxicity and mutagenicity of direct exposure to NDMA (10). Statistically significant increases in mutant fraction were observed at NDMA exposure concentrations of 5 μg/ml and 30 ng/ml for 2A3/Hol cells and 2E1/Hol cells respectively. 2D6/Hol cells were not sensitive to NDMA cytotoxicity at exposure concentrations up to 300 μg/ml. NDMA mutagenicity in 2D6/Hol cells was not examined.

Mutagenicity of NNA and NNN

In order to determine whether CYP2D6 activates other tobacco smoke-derived nitrosamines, we examined the mutagenicity and cytotoxicity of NNN and NNA to control cells and 2D6/Hol cells. The results are presented in Figure 5. NNN was non-mutagenic and non-cytotoxic to both cell lines at exposure concentrations of 150 and 300 μg/ml. An NNA-induced, concentration-dependent decrease in relative survival and increase in mutant fraction was observed in both control cells and 2D6/Hol cells.

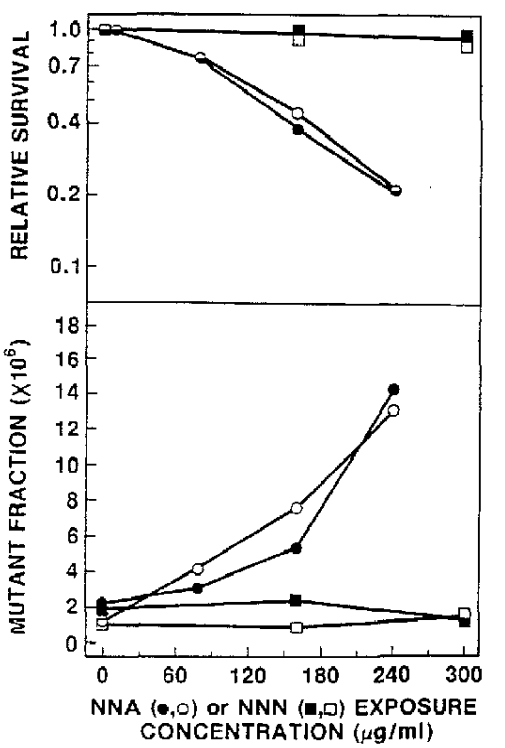

Fig. 5. Cytotoxicity and mutagenicity of NNA and NNN. Mutagenicity was determined using the same methods as described in Figure 2. Symbols are as follows: open circles, control cells−NNA; closed circles, 2D6/Hol cells−NNA; open squares, control cells−NNN; and closed squares, 2D6/Hol cells−NNN. The positive controls were 90 μg/ml NNK in 2D6/Hol cells and 5 μg/ml benzo[*a*]pyrene in control cells. The mutant fractions were 31 ± 4 × 10^{-6} and 13 ± 1 × 10^{-5} respectively.

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There were no significant differences in the responses observed in control cells and 2D6/Hol cells. The mutagenicity of NNA may be due to metabolic activation by P450s native to AHH-1 TK+/− cells. Based on these data, we conclude that there is no evidence for CYP2D6-catalyzed activation of either NNN or NNA.

Although earlier studies suggested an association between the presence of a normal CYP2D6 enzyme and smoking-associated lung cancer, there have been no reports prior to the present study that implicate CYP2D6 in the activation of any known procarcinogen present in tobacco smoke. The experiments reported herein establish that the CYP2D6 enzyme activates NNK, a procarcinogen in tobacco smoke. This same enzyme does not appear to activate NNA or NNN. The observation of procarcinogen activation by CYP2D6 suggests a mechanism for the reported linkage in the human poplation between the CYP2D6 extensive metabolizer phenotype and an increased lung cancer risk. Our study does not establish that NNK in the tobacco smoke-derived procarcinogen responsible for the apparent increased lung cancer risk. Several additional P450s—CYP1A2, CYP2A3 and CYP2E1—activate NNK. The relative abundance of these enzymes in the target cells in human lung is at present unknown. Also, the relative sensitivity of the different enzymes to inhibitors of NNK metabolism such as isothiocyanates (27) is presently unknown. Therefore, the relative importance of CYP2D6 to overall NNK activation in the target cells cannot be deduced at this time.

A number of additional procarcinogens in tobacco smoke other than NNK may contribute to the increased risk of extensive metabolizers to lung cancer. The cell line described herein can be used to determine the capability of CYP2D6 to activate enzymatically other procarcinogens. Moreover, the present study firmly establishes that CYP2D6 enzymatically converts the procarcinogen NNK to its active form. The 2D6/Hol cell line, in conjunction with isogenic control cells and cells expressing other human cytochrome P450 provide a useful model system for the study of the importance of CYP2D6 in human cancer risk.

Acknowledgements

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